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## Stress and reactivity in three Italian chicken breeds

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### ABSTRACT

Heterophil to lymphocyte ratio, plasma corticosterone and tonic immobility test, plumage and leg score (Food Pad Dermatitis, FPD) are frequently used as indicators of welfare in chickens. Three traditional Italian chicken breeds (60 birds/group) were studied: Valdarnese Bianca (VB), Bionda Piemontese (BP) and Robusta Maculata (RM). At 80 days of age, reactivity was tested through Tonic Immobility (TI) and plumage and leg score tests. Immediately after phenotypical and behavioural tests, the blood samples were collected from the ulnar vein of chickens. TI duration was not significant among the breeds (VB  $72.2 \pm 8.3$  s; BP  $95.2 \pm 9.0$  s; RM  $76.5 \pm 8.6$  s; mean  $\pm$  SEM). RM and BP needed a mean number of induction significantly higher than VB (RM =  $1.84 \pm 0.1$ ; BP =  $1.96 \pm 0.1$ ; VB =  $1.38 \pm 0.1$ ;  $p < 0.0001$ ). The highest corticosterone mean concentration ( $3.4 \pm 0.23$  ng/mL) was found in the VB breed, whereas the lowest ( $1.71 \pm 0.21$  ng/mL) was found in BP birds. The heterophil to lymphocyte (H/L) ratio was significantly ( $p < 0.0001$ ) higher in the VB breed ( $0.86 \pm 0.03$ ) than BP ( $0.78 \pm 0.02$ ) and RM ( $0.75 \pm 0.03$ ). In all birds neither plumage damages nor FPD were found. In conclusion, the three breeds showed the typical reactivity of rural breeds adapted to alternative and organic rearing systems; the anti-predatory response, the plasma corticosterone concentration and the H/L ratio could be considered a positive adaptation indicator.

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Italian chicken breeds; H/L ratio; plasma corticosterone; stress; tonic immobility test

## Introduction

Stress and fear may have deleterious effects on performances and on meat quality of birds (McKee & Sams 1997; Mashaly et al. 2004). For that reason these topics have received much attention in the poultry industry in recent years due to the growing concerns for welfare. Heterophil to lymphocyte (H/L) ratio and plasma corticosterone concentration are widely used as stress evaluation criterion in poultry (Debut et al. 2005; Franciosi et al. 2005; Cirule et al. 2012) and are well known as physiological indicators of welfare (Rogers et al. 2015; Alm et al. 2016). The H/L ratio is a more reliable indicator of mild to moderate stress than plasma corticosterone (Maxwell 1993). Corticosterone is released from the adrenal cortex and is characterized by a wide range of function: behavioural styles regulation, metabolic patterns, immune and endocrine functions and insurance of adequate coping strategy and well-being (Marelli et al. 2010).

Fear towards humans is usually evaluated with the Tonic Immobility (TI) test, that was used to investigate a fear response to manual restraint of the animal, which can be observed when the bird is set on its back in a U-shaped cradle, and hold down for 10s in a quiet environment. Upon release, the bird will stay more or less time on its back until self-righting; the measurement of the time needed before self-righting has been used to select divergent hen lines, showing either short or long TI. Selection for short TI has been accompanied by reductions in fearfulness, without changes in growth (Ferrante et al. 2005; Boissy & Erhard 2014).

Plumage score and foot pad dermatitis (FPD), are as well accepted by the veterinarians, researchers and farmers as welfare indicators. Plumage score is one of the criteria to be used in order to verify the incidence of feather pecking: it is relatively simple to measure and is heritable (Kjaer et al. 2001; Chapuis et al. 2008). FPD, also known as pododermatitis or 'foot burn', is a

condition characterized by lesions on the feet of poultry and it is widely recognized to reduce broiler performance and carcass yields and to have a negative effect on other welfare aspects (Elson 2015).

The animal biodiversity, and in particular poultry from slow growing breeds, can be a good answer to the increased demand for traditional agriculture products, linked to the territory and to the natural cycles of the seasons. In this view, the purpose of our study was to evaluate stress and reactivity of three slow-growing Italian chicken breeds (Valdarnese Bianca, Bionda Piemontese, Robusta Maculata) in response to handling and isolation. The results may enhance the knowledge about the capability of autochthonous breeds to adapt to a natural environment.

## Materials and methods

### Animals and husbandry

Three traditional Italian chicken breeds were studied: Valdarnese Bianca (VB), Bionda Piemontese (BP) and Robusta Maculata (RM). VB can be considered the only Italian meat-type chicken breed (Rooster mean weight 3.1–3.5 kg), originates from Tuscany, central Italy. The plumage is completely white with metallic golden shadows, the comb is well developed, red and erected in the rooster while side bent in the hens; the wattles are very long and bright red, skin and legs are bright yellow. BP and RM are dual-purpose breeds of chicken. BP (Rooster mean weight 2.5–2.8 kg; mean eggs/year 190) originates from Piemonte, region of north-western Italy, while RM (Rooster mean weight 4–4.5 kg; mean eggs/year 150) was created between 1959 and 1965 at the Stazione Sperimentale di Pollicoltura, Veneto, north-east of Italy. This is a chicken with buff plumage and black, blue or white tail. Skin, shanks and beaks are yellow; the comb bent on side in hens.

The RM has white and black feathers. In the one day old chicks, white is present only in small spots and the rest of the body is dark except for the belly. Adults, however, have the silver mantle and the white body with black spots.

One day old chicks (mixed sex, sex ratio 1:1) have been grouped by breed in three pens: 60 chicks each. Birds were singularly marked with a wing tag. The birds of the three breeds were reared in standard condition at the same density (10 birds/m<sup>2</sup>) in the same facility to avoid environmental effects on recorded behavioural data. The pens dimensions were 3 m (length) × 2 m (width) × 3 m (height), located indoor in the experimental facilities of the Faculty of Veterinary Medicine of the University of Milan. The initial room

temperature was set at ~32 °C and then gradually reduced based on normal management practices until reaching 21 °C on 28 days of age and with RH ranging from 65 to 75%. During the first week, a supplied light was switched on to have a photoperiod of 23:1, and then natural light was provided in all pens.

Pen floor was covered with wood shaving litter (10 cm deep) and every pen was equipped with one infra-red brooder. A standard broiler chicken pelleted diet was fed; water was *at libitum* available.

### Tonic immobility test

At 80 days of age, all the birds were tested for reactivity through Tonic Immobility. The test was carried out in a separate room close to the home house. The birds were gently moved from its home pen to the adjacent test room; this room was isolated from the house in order to avoid the effects of other bird's noises and interaction. The birds were placed on its back in a wooden cradle and restrained for 10 s. The time needed for the bird to right itself was recorded (up to a maximum of 3 min, according to the methodology used by Ferrante et al. (2005).

### Plumage and leg score

Immediately after TI test, seven body areas of each animal (comb, neck, wings, back, belly, cloaca and tail) were scored for the feather condition, the ranging scale was from 1 (plumage very damaging) to 4 (plumage not damaging), in agreement with Tauson et al. (2005). Foot pad conditions (FPD) were scored too (Hoffmann et al. 2013).

### Blood sampling

Stress response to handling and isolation in all birds has been determined at 80 days of age. In order to stress animals, after TI test and plumage and FPD scoring (handling), each bird was isolated for 1 min in a wooden box with a lid 29 × 39 × 29 cm (w × l × h), afterwards the sliding door was opened. Blood samples were collected from the ulnar vein of chickens at the same day and conditions, at 80 days of age. Sodium heparin was used as an anticoagulant, and blood samples were collected in 3-mL syringes. The samples were refrigerated and centrifuged at 500 × *g* for 15 min at 5 °C.

Plasma samples were collected, frozen at –20 °C in micro centrifuge tubes until analysis and stored (–80 °C). A blood smear was made for each pullet concurrently with blood sampling. The blood smears were

allowed to dry and smears were later stained using a leukostat staining kit (Fisher Laboratory Products, Pittsburgh, PA). The first 100 heterophils and lymphocytes were counted on each smear under an oil immersion lens of 1000 $\times$  total magnification. The ratio counted was recorded as the H:L. Plasma corticosterone analyses were conducted according to the methods of Davis et al. (2000) using radioimmunoassay kits (Diagnostic Products Corp., Los Angeles, CA). Pooled plasma CORT levels, as determined by the radioimmunoassay kits were used to calculate the intra-assay CV (4.3%) and the inter assay CV (6.0%). Plasma hormone (CORT) was measured by radioimmunoassay using a commercial kit (Franciosini et al. 2005).

### Statistical analysis

Statistical analysis were performed by the analysis of variance using General Linear Model procedure to evaluated the effect of the breed and the sex on TI (duration and induction), corticosterone levels, and on the H/L ratio. Moreover the Spearman correlation was used on corticosterone, H/L and TI duration and number of induction (SPSS 2013), to evaluate relationship between variables.

The plumage condition and FPD lesion were not included in the model as the breeds showed almost no variability for these traits.

### Results and discussion

No effects of sex on TI duration and number of induction were found in all breeds; also Elfwing et al. (2015) did not found any effect of sex on TI duration and number of inductions.

Significant differences among breeds were recorded during the Tonic Immobility test as showed in Figure 1. TI evaluates fearful behaviour in the chicken and represents a defensive reaction that can be used as criterion for measuring the wellbeing and stress levels of the birds (Campo et al. 2006). TI duration didn't show any differences among the breeds (VB  $72.2 \pm 8.3$  s; BP  $95.2 \pm 9.0$  s; RM  $76.5 \pm 8.6$  s; mean  $\pm$  SEM). These results differ to the ones obtained by De Marco et al. (2013) who found differences related to the breeds and higher values in Bionda Piemontese (TI mean duration  $281 \pm 25.66$  s). These differences may be due to a comparison of two local breeds with a commercial hybrid which is recognized to have a higher TI reaction. Moreover, they use only females and the animals were older than ours.

The TI duration of all the three breeds, report results in agreement with those found out in slow growing breed and always lower in respect of commercial meat hybrids (Ross - Castellini et al. 2002; Perai et al. 2014) and laying hens (Hy Line Brown - Mugnai et al. 2011). Cockrem (2007) found similar results attributing them to individual responses of each bird.

The results of the number of inductions presented significant differences among the breeds (Figure 1): RM and BP needed a significantly higher number of inductions than VB to reach the immobility (RM =  $1.84 \pm 0.1$ ; BP =  $1.96 \pm 0.1$ ; VB =  $1.38 \pm 0.1$ ;  $p < 0.001$ ; mean  $\pm$  SEM).

VB registered the lowest number of induction and BP the highest duration of immobility, due probably at the higher fear towards humans in both breeds. These breeds seem to have maintained ancestral characteristics and anti-predatory behaviours more than RM; poultry breeds with short lasting freezing responses are more prone to be killed by predators (Abea et al. 2013). On the contrary RM breed seems to show

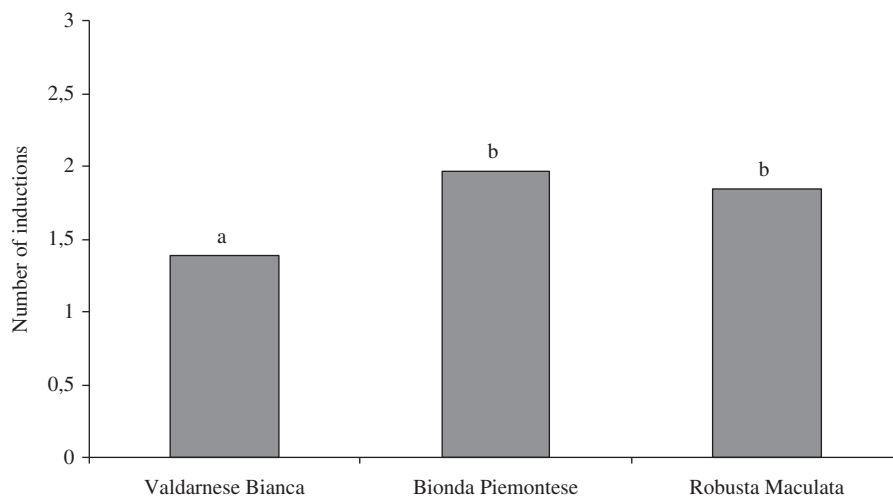


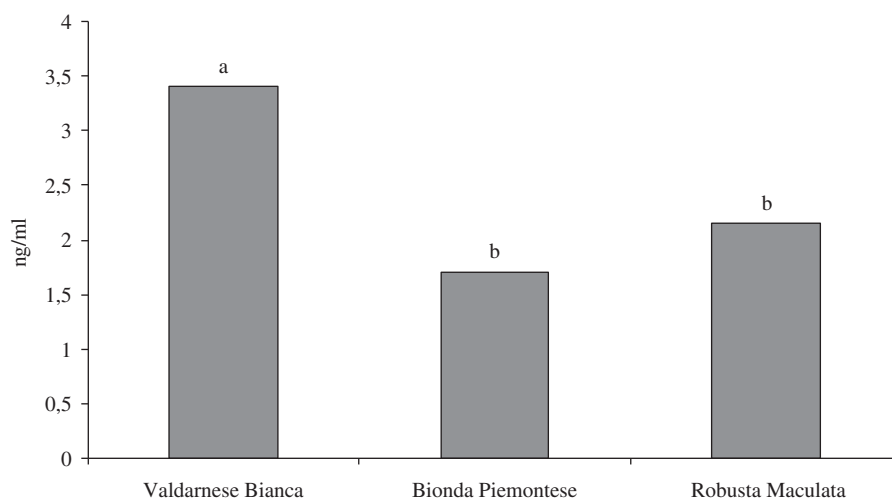
Figure 1. Mean number of inductions of the Tonic Immobility test among the breeds (different letter for  $p < 0.0001$ ).

reactivity more similar to commercial hybrids (Campler et al. 2009).

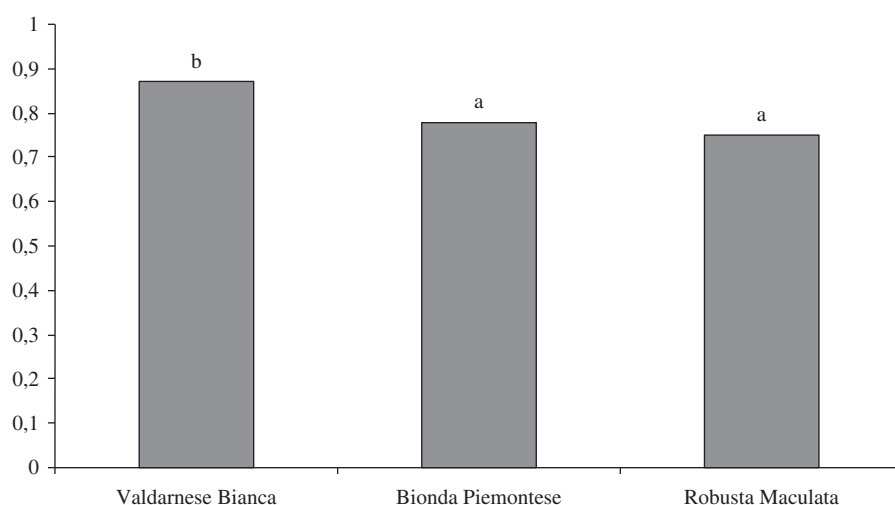
The highest CORT mean concentration ( $3.40 \pm 0.23$  ng/ml) was found in the VB breed (Figure 2), whereas the lowest ( $1.70 \pm 0.21$  ng/ml) was found in BP birds. In RM birds, the CORT concentration recorded was intermediate, average  $2.15 \pm 0.19$  ng/ml. Significant effect of the breed on blood CORT levels was found between VB vs. RM and BP ( $p \leq 0.001$ ). Corticosterone is the principal adrenocorticoid in the peripheral blood and elevated blood corticosterone concentrations represent stress indicator in birds (Freeman 1983; Mc Farlane & Curtis 1989; Delezie et al. 2007). Furthermore, the effect of the genetic strains on plasma corticosterone levels was studied by different authors (Fraisie & Cockrem 2006; Chapuis et al. 2008). Our results show significant differences in plasma concentrations in the three studied breeds within a species-specific physiological range of 1–5 ng/ml. Breed differences could be due to birds' genetic make-up. The adrenal system is fundamentally involved in animal adaptability control. Corticosterone levels affect stress-induced responsiveness and can protect or destroy the coping ability of the organism. High levels of corticosterone might be a part of the organism's defence against stressors (Marelli et al. 2010). Hazard et al. (2008), in two divergent selection for duration of tonic immobility Japanese quail chicks (*Coturnix coturnix japonica*), found that this type of genetic selection has affected the Hypothalamic–Pituitary–Adrenal (HPA) axis response to stress. One physiological component of the stress response in vertebrates is the activation of the HPA axis, which results in the release of corticosteroids (i.e. corticosterone in birds) from the adrenal glands (Siegel 1971; Scott et al. 1983; Harvey & Hall 1990; Jones et al. 1994; Romero & Wingfield 2001;

Canoine et al. 2002). Corticosteroids contribute to the re-establishment of homeostasis via negative feedback mechanisms on hypothalamus and/or pituitary structures decreasing HPA axis activation (Schulkin et al. 1994, 1998; Canoine et al. 2002). They act to facilitate adaptive behavioural responses by providing the metabolic requirements for flight or fight responses (reviewed in Sapolsky et al. 2000). The males recorded significant higher corticosterone levels in all the breeds (VB males  $3.78 \pm 1.8$  females  $2.84 \pm 1.3$ ; BP males  $2.20 \pm 1.9$  females  $1.19 \pm 0.99$  and RM  $2.6 \pm 1.74$  females  $1.65 \pm 0.98$ ), these results agree with the findings by Elfving et al. (2015) who hypothesized that in male birds an early stress caused a higher HPA-reaction.

As showed in the Figure 3, the H/L ratio was significant higher in the VB ( $0.87 \pm 0.03$ ) than in the BP ( $0.78 \pm 0.02$ ) and in the RM ( $0.75 \pm 0.03$ ). Concerning haematic traits, heterophils increase and lymphocytes decrease when chickens are stressed, so that the ratio between them is a good index of response to a stressor. These findings disagree with those reported by De Marco et al. (2013) who found in BP a mean value of  $0.40 \pm 0.02$ ; probably this can be due to the older age of tested animals. A part from the breed the H:L parameter can be affected by a variety of factors including sex, and age of birds (Rogers et al. 2015). Bayyari et al. (1997) reported that lymphocyte numbers were lower in a line of turkeys selected for heavier BW than in a line selected for increased egg production. The differences for heterophils, lymphocytes and the ratio between them observed in our experiment may be linked to breed differences. In contrast, De Marco et al. (2013), found in BP hens, reared in free-range, a lower H/L ratio. These differences can be attributed to age (our birds were 80 d old vs. 26 wks), sex (mixed sex vs. hens) and housing condition (indoor vs. free-range).



**Figure 2.** Mean plasma corticosterone concentration (ng/ml) among the breeds (different letter for  $p < 0.0001$ ).



**Figure 3.** Mean of ratio Heterophil to Lymphocyte among the breeds (different letter for  $p < 0.0001$ ).

Campo and Davila (2002), estimated the heritability of the heterophil to lymphocyte ratio and evaluated the effects of age, sex, and crossing on that ratio, in a Spanish breed of chickens; the males had significantly greater H/L ratio than females at the onset of sexual maturity and as adults, the females showed a significant effect on heterophil and lymphocyte numbers. Moreover, focusing on environmental conditions, it has been reported that H/L ratio of BP in the free range is similar to that of organic Ancona hens (Mugnai et al. 2011), and that of Ancona control group (under standard rearing conditions) is similar to our BP indoor. This result indicates that genotype-environment interactions are likely to be of high importance in evaluating animal stress responses. Gross & Siegel (1983) suggested that reference values for the heterophil to lymphocyte ratio of about 0.2, 0.5 and 0.8 are characteristic of low, optimal and high degrees of stress, respectively. On the basis of this affirmation and accordingly to Shini et al. (2008), Valdarnese Bianca breed was the most 'stressed' with the highest H/L values. Accordingly, in this breed, it was found the highest value of heterophil (40.86%) and the lowest value of lymphocytes (48.85%), compared with the other two breeds.

Spearman's correlation showed some interesting results (Table 1). Plasma corticosterone was negatively correlated to the TI number of induction, whereas TI number of induction was negatively correlated to the TI duration; H/L ratio was significantly positively correlated to the TI duration in the Valdarnese Bianca. In this breed, the results obtained were in agreement with several authors (Jones et al. 1988; Beuving et al. 1989; Fraisse & Cockrem et al. 2006), that found positive relationship between circulating corticosterone, fear-induced behaviour (number of induction) in

**Table 1.** Spearman's correlation ( $\rho$ ) among Cort (corticosterone), TI ind (Tonic Immobility number of inductions), TI dur (Tonic Immobility duration), Ratio H/L (ratio Heterophil to Lymphocyte).

Breed	Cort	TIind	TI dur	Ratio H/L
Valdarnese Bianca				
Cort	1	-0.117	-0.082	-0.053
TIind		1	-0.035	0.092
TI dur			1	0.282*
Ratio H/L				1
Bionda Piemontese				
Cort	1	-0.132	-0.008	0.093
TIind		1	-0.386**	0.142
TI dur			1	-0.006
Ratio H/L				1
Robusta Maculata				
Cort	1	-0.017	0.041	0.015
TIind		1	-0.094	-0.004
TI dur			1	-0.002
Ratio H/L				1

\*\* $r < 0.01$ ; \* $r < 0.05$ .

several avian species. These authors suggested that chronic elevations of plasma corticosterone not only alter the haematological profile but may also predispose birds to react more fearfully to alarming stimulation (Jones et al. 1988). Ghareeb (2010), found positive correlation between the duration of tonic immobility with fear and serum corticosterone.

The plumage conditions in all the body areas were scored around 4 (plumage not damaged; VB  $3.98 \pm 0.07$ ; BP  $3.98 \pm 0.09$ ; RM  $3.96 \pm 0.1$ ). Regarding feathers conditions, it is largely documented that feather pecking is the most responsible of feather conditions and is a multi-factorial problem affected by the genetic background of the birds (Nicol et al. 1999; Bilcik & Keeling 2000; Grams et al. 2015). No difference in FPD score was registered. In fact, all studied birds are light breeds, which did not show feet problems caused by their high body and breast weights which



forced the animals to bed rest and reduced their mobility. FPD identification is recognised important not only to highlight a potential welfare issue for birds, but it can also alert to potential problems with litter management or imbalances in the feed (Bassett 2009).

## Conclusions

The three studied breeds showed a typical reactivity of rural breeds for alternative and organic rearing systems; in fact the anti-predatory response, used by the individuals in challenges situations, the plasma corticosterone concentration and the H/L ratio could be considered positive adaptation ability useful to maintain stress in the admitted physiological range. These results may be useful to those who want to rear these breeds. In fact to avoid unnecessary stress and welfare problems it can be necessary to properly manage these animals in accordance with their genetic make-up. These animals can be well adapted to natural environment but they need, for example, natural or artificial shelter and system to avoid the stress of predation to which they are very reactive.


## Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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